

Effect of female reproductive diapause on *Drosophila melanogaster* innate immune defense

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Abstract: *Drosophila melanogaster* females undergo reproductive dormancy, or diapause, under conditions of low temperature and short daylight hours. This stage is characterized by non-vitellogenic ovaries, increased lipid stores, decreased senescence, and increased resistance to stress. My study tests whether diapause affects resistance to bacterial pathogens and whether an infection influences entry into diapause. I hypothesize that diapause and immunity are reciprocally connected, with diapause increasing resistance to infection and infection increasing the probability of entry into diapause. I have preliminarily found that *D. melanogaster* genotypes with higher propensity to enter into diapause sustain decreased pathogen load after infection. Previous studies show that insulin signaling may regulate diapause, and that the Toll signaling pathway of the innate immune response disrupts insulin signaling. Future experiments will determine whether the relationship between immunity and diapause is mediated by regulation of insulin signaling, and what roles the genes regulating diapause or immunity play in regulating the insulin pathway. The results of my study provide the first evidence that, in addition to decreased senescence and increased stress resistance, the diapause state also increases resistance to bacterial infection.

Keywords: *Drosophila melanogaster* | diapause | immunity | *couch potato* gene | life history

Introduction:

Populations that exist over a broad geographic range must be able to deal with a variety of local environmental challenges. Often, these adaptations are pleiotropic, and as a result, can cause trade-offs between various systems in the organism. To cope with harsh winters at higher latitudes, *Drosophila melanogaster* undergoes reproductive dormancy, or diapause, a state that is marked by a suite of physiological changes. The goal of my study is to see if the dormant state affects or is affected by the immune defense. While the effects of diapause on longevity,

fecundity, and resistance to stress have been investigated, nothing is known about the effect of diapause on immune defense. Understanding the impacts that diapause and defense have on each other will show a new aspect of a classic life history trade-off between maintenance of somatic tissues and reproduction. It will also allow for more informed predictions of the effects of climate change on insect populations. To answer this question, I test whether flies in diapause show a change in resistance to infection by bacterial pathogens and whether infection affects entry into diapause.

Drosophila melanogaster female reproductive dormancy is a state that is marked by arrest of oocyte development before vitellogenesis and accumulation of lipids and glycogen stores. Short days and low temperatures characteristic of the fall and winter months of northern latitudes are the triggers for entry into the diapause state (Saunders et al. 1989). This diapause state is associated with increased stress resistance and decreased senescence (Tatar et al. 2001).

The genes responsible for regulating diapause are still being discovered – the *couch potato* gene (*cpo*) explains a large proportion of the variation in diapause propensity in natural populations of *D. melanogaster*. The two alleles of *cpo* vary both spatially and temporally with the incidence of diapause (Schmidt et al. 2008). Low expression of *cpo* results in a higher probability of diapause entry, or a high-diapause phenotype, while high expression results in the opposite, or low-diapause, phenotype. Ecdysone, an insect molting hormone, has been implicated in regulating diapause – it is at low levels during diapause, but an injection of ecdysone into diapausing flies can bring about an increase in vitellogenesis (Richard et al. 2001). The expression of *cpo* is potentially hormonally regulated by ecdysteroids through ecdysone response elements, which gives more evidence for the role of ecdysone in diapause regulation (Schmidt et al. 2008). In addition to ecdysone regulation, disruptions in juvenile hormone (JH) are implicated in the initiation of diapause in *D. melanogaster* and application of JH has been shown to

terminate reproductive diapause (Hahn & Denlinger 2011). In a separate study, wild-type levels of JH and ecdysteroids were insufficient to induce vitellogenesis in insulin signaling deficient flies (Richard et al. 2005). Incubation of diapausing ovaries with 20-hydroxyecdysone also failed to induce vitellogenesis (Richard et al. 2001). This evidence points to another factor that is required for vitellogenesis and possibly diapause, as the absence of vitellogenesis is a key characteristic of the diapause state. Richard et al. propose that ovary endogenous insulin signaling is required for vitellogenesis to occur (2005). Disruption of insulin signaling in *D. melanogaster* results in a state that is similar to diapause (Hahn and Denlinger 2011). Therefore, insulin signaling may also play a key role in regulating the onset of diapause.

Activation of the Toll innate immunity signaling in *D. melanogaster* decreases insulin signaling (DiAngelo et al. 2009). It follows that there may be a reciprocal connection between the Toll immune response and diapause through regulation of insulin signaling. My hypothesis is that the *cpo* lines that express a high rate of entry into diapause will have increased resistance to bacterial infection and that bacterial infection will increase entry into diapause. This increase in resistance might occur through downregulation of insulin signaling, the increased storage of nutrients, and the removal of resources from reproduction. Similarly, I predict that activation of the immune signaling system would decrease insulin signaling and facilitate entry into diapause.

I carried out infection experiments on genotypes expressing different levels of *cpo*, and thereby different propensity for diapause. Under my hypothesis, I predicted that the genotypes with a higher proportion of flies in diapause would have higher resistance to infection. I also predicted that flies that are infected would express a higher rate of entry into diapause. I tested both a gram-negative and a gram-positive pathogen in my experiments as the immune response for the two types of pathogens works through different pathways – Imd for gram-negative and Toll for gram-positive (DiAngelo et al., 2009). By using the two types of pathogens, I tested

whether the connection between diapause and defense extends to just the Toll signaling pathway, as predicted by regulation of insulin signaling, or to both Toll and Imd pathways. My experiments showed a positive correlation between diapause propensity and resistance to infection across both pathogens. These findings will flesh out our understanding of the effects of diapause on various systems in *D. melanogaster* and other invertebrates. Exploring the mechanism of action of the connection between diapause and immunity by way of insulin signaling pathway will also give an insight into how the life history trade-off between somatic maintenance and reproduction work on a molecular level, an aspect of life history trade-offs that is often unknown.

Methods:

Generation of high- and low-diapause lines

High- and low-diapause genotypes were generated in order to compare bacterial loads. High-diapause genotypes have a higher probability of a fly entering diapause when placed at diapause conditions. Initial infection experiments were run using the *cpo*^{BG02810} (*cpo*^{hypo}) line, a strong P-element hypomorph, and *cpo*^{P3} (*cpo*^{wt}), a line with a precise excision of that P-element. Since *cpo*^{hypo} is not viable in a homozygous state, both lines were crossed to *cpo*^{v3}, a weak P-element hypomorph, to generate genotypes that can be compared in terms of diapause propensity and *cpo* expression. *cpo*^{hypo} / *cpo*^{v3} (*cpo*^{hypo/v3}) is a high diapause genotype with around 90% entering diapause at 11°C and 10L:14D light regime and *cpo*^{wt} / *cpo*^{v3} (*cpo*^{wt/v3}) is a low diapause genotype with approximately 45% entering diapause with that same regime (SI: Schmidt et al. 2008).

Another infection experiment was run with *cpo*^{3.42} (*cpo*^{del}), which has a *cpo* deletion, and *cpo*^{3.20} (*cpo*^{dupl}), which has a tandem *cpo* duplication. Both *cpo*^{del} and *cpo*^{dupl} were crossed to

cpo^{v3}, *cpo^{del/v3}* genotypes express diapause at a higher rate (>70% flies in diapause) than *cpo^{dupl/v3}* genotypes (<25% of flies in diapause). *cpo^{wt}*, *cpo^{hypo}*, *cpo^{del}*, *cpo^{dupl}* have the same w⁶³²⁶ genetic background, as well as *cpo^{v3}* with the exception of its 3rd chromosome (Schmidt et al., 2008).

All flies were grown on standard Cornell media (8.3% glucose, 8.3% Brewer's yeast, and 1% agar, plus 0.04% phosphoric acid and 0.4% propionic acid added to inhibit microbial growth in the food) at 24°C and 12L:12D light cycle.

Inducing and scoring for diapause

To induce diapause, flies were placed in food vials and kept at 11°C and 10L:14D light cycle for 4 weeks, here onwards referred to as diapause conditions. The diapause phenotype was assayed by dissection of the abdomen in Phosphate Buffered Saline (PBS). The fly was said to be diapausing if both ovaries showed no vitellogenesis (ovary arrested before stage 8), and non-diapausing if there was vitellogenin in either ovary (stage 8 and later) (Schmidt et al. 2008). The stage of each ovary was determined by the most advanced ovariole present, as there is lack of developmental synchrony in the ovarioles. This provides a very conservative method of characterizing diapause (Saunders et al. 1989).

Pinprick infections and determination of bacterial load

Female virgin flies were infected with *Providencia rettgeri*, a gram-negative bacteria, and *Enterococcus faecalis*, a gram-positive bacteria. The liquid bacterial cultures were created by inoculating LB liquid media with a single colony and incubated at 37°C in a shaking incubator for 18-24 hours. The A₆₀₀ for the 24°C infections was 0.532 ± 0.002 for *P. rettgeri* and 0.356 ± 0.027 for *E. faecalis*. The A₆₀₀ for the 11°C infections was 0.920 ± 0.078 for *P. rettgeri* and

0.397 \pm 0.029 for *E. faecalis*. The infection was carried out by anesthetizing the fly for no more than 10 minutes using CO₂ and pricking the fly's thorax with a 0.15mm insect pin (Fine Science Tools) dipped in the liquid bacterial culture. For experimental controls, flies were pricked with a sterile needle or kept on CO₂ without being pricked. The flies that were to be assayed for bacterial load were continually kept on ice. To determine bacterial load, 3 flies were homogenized in an Eppendorf tube containing 500 μ L of LB media. 50 μ L of the resulting suspension was plated onto a LB agar plate using a WASP 2 spiral plater (Microbiology International, Bethesda, MD, USA) at various dilutions. Plates were kept overnight at 37°C and the Colony Forming Units (CFU) per fly were counted using ProtoCOL plate counting software (Microbiology International).

Infection of Canton S females at diapause conditions

This experiment was carried out to confirm that the bacteria would be able to grow at diapause conditions. Three to seven day old Canton S female flies were infected using the protocol described above and put at diapause conditions for a 6 day period. Bacterial loads were measured on the initial infection day within 3 hours of infection, or the initial time point, (no dilution) and then every other day (*P. rettgeri*: 1:10, 1:1000, 1:1000 dilutions, respectively; *E. faecalis*: 1:10, 1:10, 1:10 dilutions, respectively). Survival was recorded daily over two and a half week period after infection.

Infection of $cpo^{wt/v3}$ and $cpo^{hypo/v3}$ virgins at 24°C

This experiment tested for an effect of genotype on the immune response that is independent of diapause conditions. The flies were reared and the experiment carried out at 24°C and 12L:12D light regime, which mimics the ancestral tropical environment of *D. melanogaster*.

Three to five day-old $cpo^{hypo/v3}$ and $cpo^{wt/v3}$ female virgins were infected as per the outlined protocol and the bacterial load was measured at the initial time point (no dilution) and at 24 hours (*P. rettgeri*: 1:1000 dilution; *E. faecalis*: 1:100 dilution). Survival was measured over a five-day period.

Infection of $cpo^{wt/v3}$ and $cpo^{hypo/v3}$ virgins at diapause conditions

In order to test for an effect of diapause propensity on defense, an infection experiment was run at diapause conditions of 11°C and 10L:14D. The $cpo^{hypo/v3}$ and $cpo^{wt/v3}$ were collected 8 hours post-eclosion and put at diapause conditions for 4 weeks. At the end of 4 weeks, the flies were infected using the same pathogens and procedure as above, but continually kept at diapause conditions. Initial bacterial load (no dilution) and a 96-hour bacterial load (*P. rettgeri*: 1:1000 dilution; *E. faecalis*: 1:10 dilution) were measured. A sample of twenty to forty flies from each genotype was sampled and scored for diapause. Survival was measured over a two and half week period.

Infection of $cpo^{dupl/v3}$, $cpo^{del/v3}$, $cpo^{wt/v3}$, and $cpo^{hypo/v3}$ virgins at diapause conditions

This infection experiment tested $cpo^{hypo/v3}$, $cpo^{wt/v3}$, $cpo^{dupl/v3}$, and $cpo^{del/v3}$ genotypes at diapausing conditions to see whether infection of the $cpo^{dupl/v3}$ and $cpo^{del/v3}$ genotypes would yield the same results as of $cpo^{wt/v3}$ and $cpo^{hypo/v3}$ genotypes. The protocol was the same as for the second infection experiment, except that all dilutions for the 96-hour post infection load were 1:10. Survival was not measured for this infection trial.

Effect of chronic infection on diapause entry

The final infection experiment used 5-9 day old Oregon-R female flies, which were kept at 24°C and 12L:12D for the time period between eclosion and their placement at diapause conditions. The flies were infected with *P. rettgeri* or *E. faecalis*, following the same protocol and controls as the first infection experiment, and put at 11°C and 10L:14D. At the end of the 4 week period, the flies were scored for diapause. Assaying flies for the presence of bacteria at the end of the 4 week period determined the presence of a chronic infection. The percent of flies in diapause was compared between infected flies and those injured with the sterile needle or CO₂-anesthetized only.

Statistical Analysis

All statistical analyses were carried out in R version 3.0.1 (R Core Team 2013). The formula for the linear model is as follows, with genotype, bacteria, and their interaction all treated as fixed effects:

$$\ln(\text{Bacterial Load}) = \text{Genotype} + \text{Bacteria} + \text{Genotype} * \text{Bacteria} + \text{error}$$

The linear model was created using the Stats package. Survival was analyzed using the Survival package. The model for the Cox proportional hazards model is as follows with all effects fixed:

$$\text{Survival}(\text{time, event}) = \text{Genotype} + \text{Bacteria} + \text{Genotype} * \text{Bacteria} + \text{error}$$

The event is the death of a fly and all flies that did not die were censored at the end of the experiment.

Results:

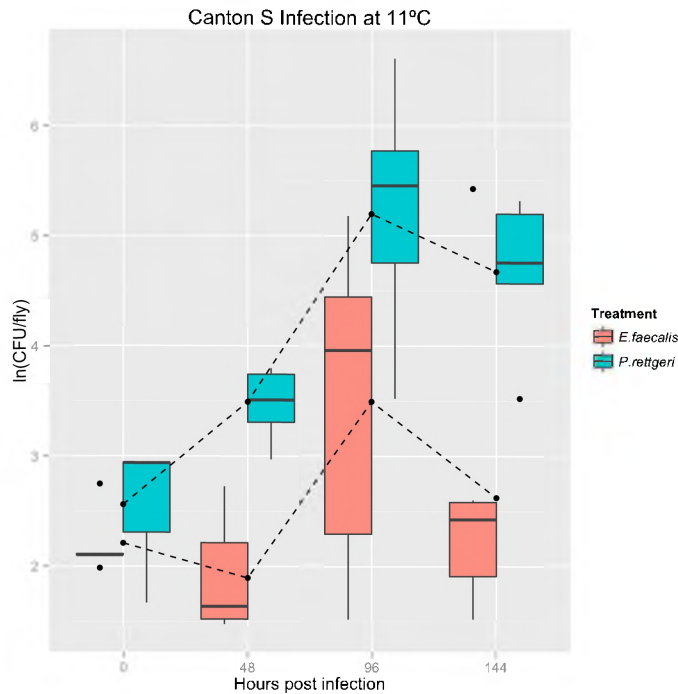
Canton S infection at diapause conditions

Canton S female flies were used to generate an infection time series at diapause conditions (Figure 1A). There is a bacterial load peak at the 96-hour mark for both pathogens, which was used as a comparison time point for the rest of the infections carried out under diapause conditions. Analysis of the survival of the three treatments using ANOVA on a Cox proportional hazards model resulted in no significant difference in mortality at diapause conditions due to infection by the two bacteria compared to sterilely wounded flies ($p = 0.43$; Figure 1B).

$cpo^{wt/v3}$ and $cpo^{hypo/v3}$ infection at 24°C and 12L:12D

Virgin $cpo^{wt/v3}$ (wild-type) and $cpo^{hypo/v3}$ (cpo hypomorph) females were infected with either *P. rettgeri* or *E. faecalis* and bacterial loads were compared at the 24-hour time point in order to see whether the genotype of the fly had an effect on resistance to bacterial infection. There was a significant effect of pathogen on the CFU/fly ($p = 0.036$), with *P. rettgeri* growing to higher loads, but neither the effect of genotype nor the effect of the interaction of genotype and pathogen were significant (Figure 2A). Flies infected with *E. faecalis* had higher mortality than those infected with *P. rettgeri*, and infected flies had higher mortality than sterilely wounded flies ($p < 0.00023$), but no genotype or interaction effects were significant (Figure 2B). The genotype of the fly had no effect on its bacterial load at the 24-hour time point or the probability of survival post-infection.

A.



B.

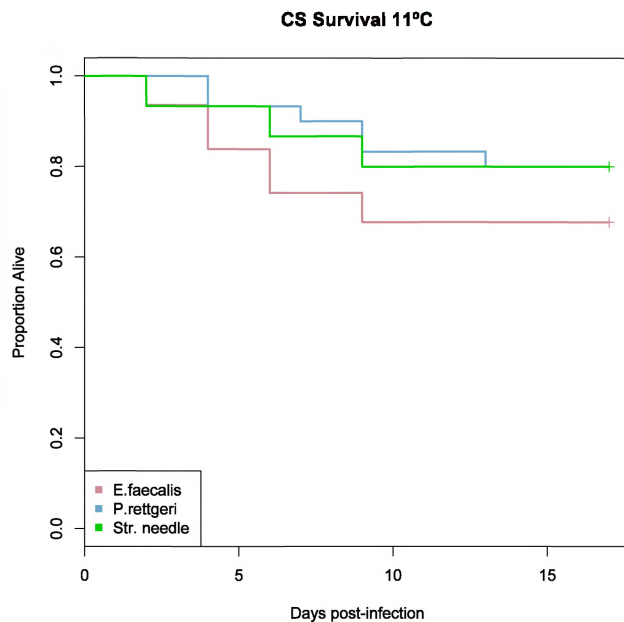
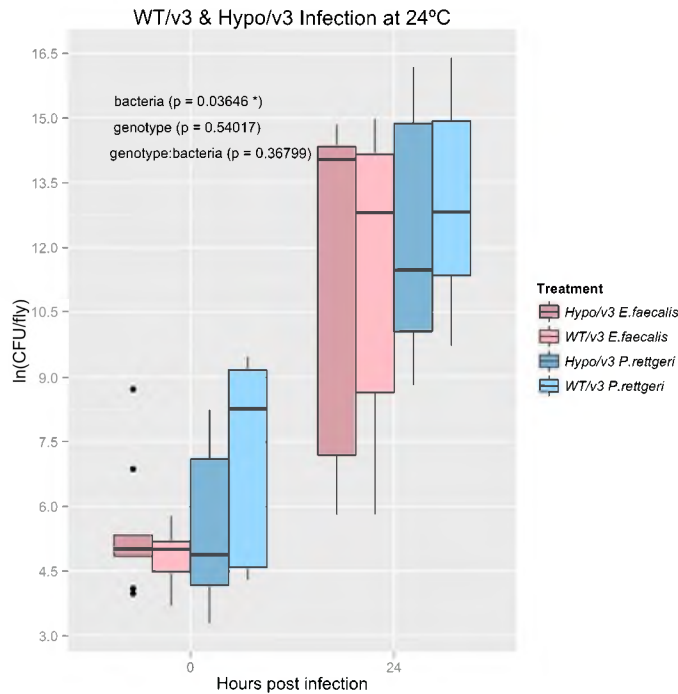


Figure 1. Infection of Canton S (CS) female flies with *P. rettgeri* and *E. faecalis* at 11°C and 10L:14D. (A) Bacterial load curve over a 6 day period. Dashed lines connect the mean bacterial load for each pathogen for that time point. Initial time point sample size (n=5); rest of time points (n=10). (B) Survival curves over a 17 day period. Sample sizes for the treatments are as follows: *P. rettgeri* (n=30), *E. faecalis* (n=30), sterile needle (n=15).

A.



B.

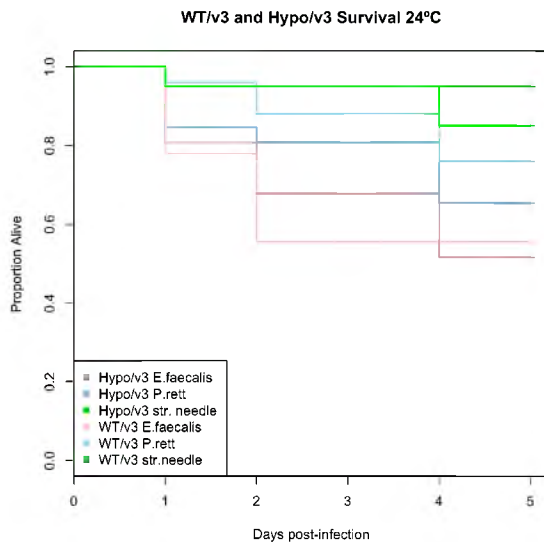
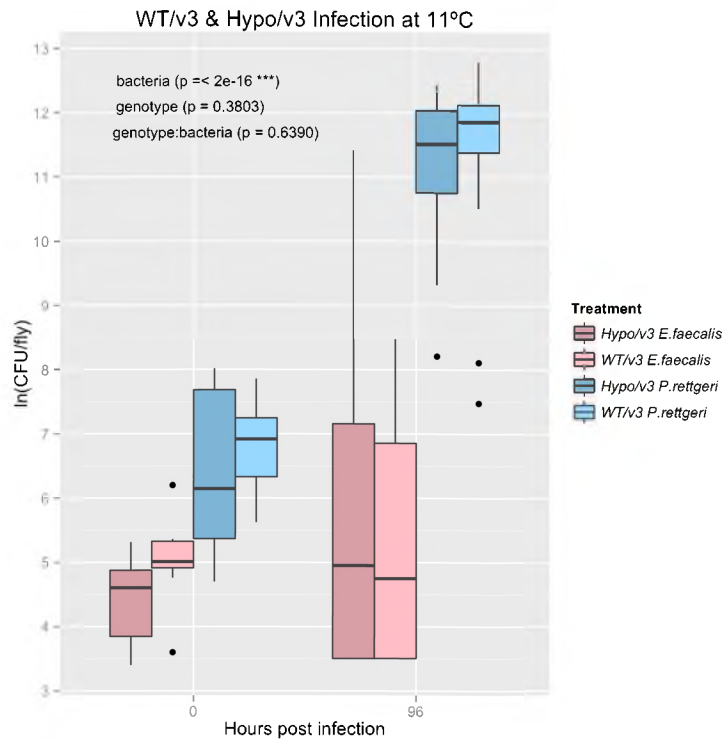


Figure 2. Infection of $cpo^{wt/v3}$ (WT/v3) and $cpo^{hypo/v3}$ (Hypo/v3) virgin female flies with *P. rettgeri* and *E. faecalis* at 24°C and 12L:12D. (A) Two replicates were combined to generate this boxplot; initial time point sample size (n = 10), 24 hour time point sample size (n = 20). P-values were generated by using ANOVA on a linear model. **(B)** Survival curve over a 5 day time period. Sample sizes are as follows: $cpo^{wt/v3}$ *P. rett* (n = 28), $cpo^{wt/v3}$ *E. faec* (n = 27), $cpo^{wt/v3}$ str. needle (n = 20), $cpo^{hypo/v3}$ *P. rett* (n = 26), $cpo^{hypo/v3}$ *E. faec* (n = 31), $cpo^{hypo/v3}$ str. needle (n = 20). CO₂ replicates were not included ($cpo^{wt/v3}$: n = 20, $cpo^{hypo/v3}$: n = 17) because no deaths occurred over the time course. P-values were generated by using ANOVA on a Cox proportional hazards model.

$cpo^{wt/v3}$ and $cpo^{hypo/v3}$ infection at diapause conditions

To establish whether diapause propensity affects resistance to bacterial pathogens, I carried out an infection experiment using high- and low-diapause lines under diapause conditions. Virgin $cpo^{wt/v3}$ (wild-type) and $cpo^{hypo/v3}$ (*cpo* hypomorph) females were kept at diapause conditions for 4 weeks and then infected with either *P. rettgeri* or *E. faecalis*. Bacterial loads were compared at the 96-hour time point established in the preliminary Canton S infection discussed above. Flies infected with *P. rettgeri* had overall higher bacterial loads ($p \leq 2 \times 10^{-16}$), but no significant effect of genotype or effect of the interaction of genotype and pathogen was observed (Figure 3A). The $cpo^{wt/v3}$ genotype had a higher proportion of flies in diapause than $cpo^{hypo/v3}$ ($p = 2.5 \times 10^{-5}$), but this had no effect on the bacterial load at the 96-hour time point or the survival post-infection at diapause conditions (Figures 3B, 4A, 4B). Infected flies did not differ in mortality from sterilely wounded flies. The observed proportion of flies in diapause differed significantly in the opposite direction ($cpo^{hypo/v3}$: 31.5%, $p = 0.00080$; $cpo^{wt/v3}$: 96%, $p = 0.00027$) from the expected proportions ($cpo^{hypo/v3}$: ~ 90%, $cpo^{wt/v3}$: ~ 45%) found by Schmidt et al. (2008) (Figure 3B), which may indicate that there is a factor affecting proper *cpo* expression in these genotypes.

A.



B.

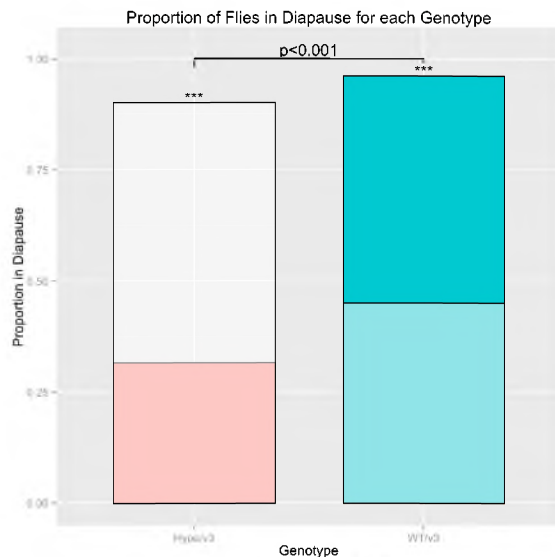
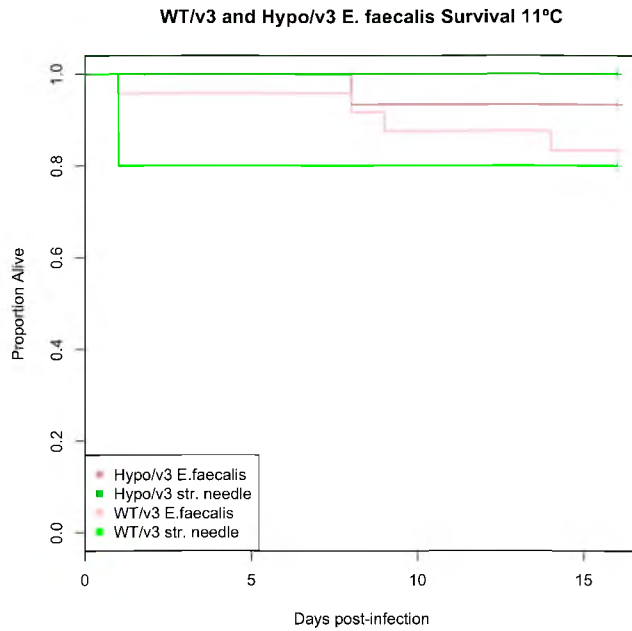


Figure 3. Infection of *cpo*^{wt/v3} (WT/v3) and *cpo*^{hypo/v3} (Hypo/v3) female virgins with *P. rettgeri* and *E. faecalis* at 11°C and 10L:14D. (A) Combined plot of two replicates; initial time point sample size ($n = 7-8$), 96-hour time point sample size ($n = 17-20$). P-values were generated using ANOVA on a linear model. **(B)** Colored bars represent the observed proportion in diapause; lighter shading/white bars represent the expected proportion in diapause (SI: Schmidt et al. 2008). *cpo*^{wt/v3} ($n = 25$), *cpo*^{hypo/v3} ($n = 19$). P-values were generated using a proportion test with Yates continuity correction on the observed proportions. Asterisks show a significant difference between the observed and expected proportion in diapause: *** ($p < 0.001$)

A.



B.

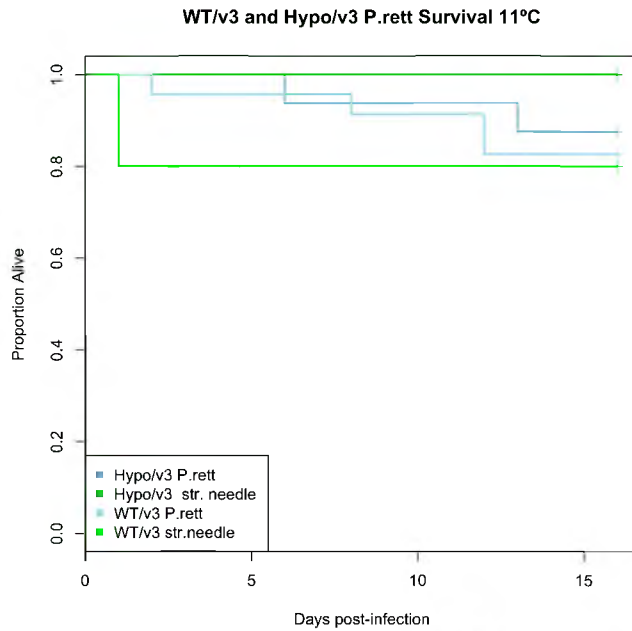
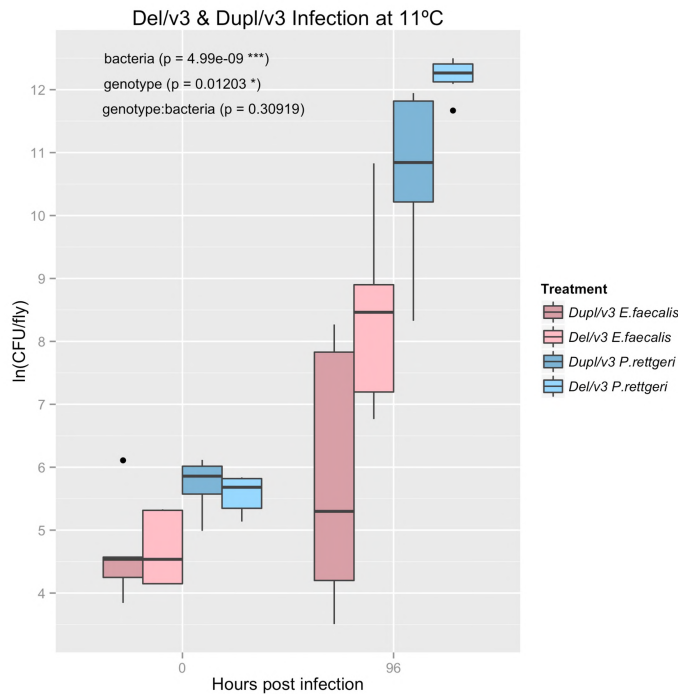


Figure 4. Survival curves for $cpo^{wt/v3}$ (WT/v3) and $cpo^{hypo/v3}$ (Hypo/v3) virgin females infected with *P. rettgeri* (*P. rett*) and *E. faecalis* (*Ent*) at 11°C and 10L:14D. (A) *E. faecalis* survival curve; $cpo^{hypo/v3}$ *E. faecalis* (n=15), $cpo^{hypo/v3}$ sterile needle (n=3), $cpo^{wt/v3}$ *E. faecalis* (n=24), $cpo^{wt/v3}$ sterile needle (n=5). (B) *P. rettgeri* survival curve; $cpo^{hypo/v3}$ *P. rettgeri* (n=16), $cpo^{wt/v3}$ *P. rettgeri* (n=23). Sterile needle controls are the same as in (A).

cpo^{del/v3} and cpo^{dupl/v3} infection at diapause conditions

Since the first pair of high- and low-diapause genotypes showed no effect of diapause propensity on resistance, an infection experiment was run on another pair of genotypes in order to see if the lack of effect was ubiquitous. Virgin *cpo^{del/v3}* (*cpo* deletion) and *cpo^{dupl/v3}* (*cpo* duplication) females that were kept at diapause conditions for 4 weeks were infected with *E. faecalis* and *P. rettgeri* and bacterial loads were compared at the 96-hour time point. This infection was done in parallel with a replicate infection of virgin *cpo^{wt/v3}* and *cpo^{hypo/v3}* females. Pathogen ($p = 5 \times 10^{-9}$) and genotype ($p = 0.012$) both affect bacterial load. *cpo^{dupl/v3}* virgins had lower bacterial loads across both pathogen treatments, and *P. rettgeri* infected flies had overall higher bacterial loads (Figure 5A). The *cpo^{dupl/v3}* genotype had a higher proportion in diapause than *cpo^{del/v3}* ($p = 0.027$). The observed values for *cpo^{dupl/v3}* were significantly different (85.7%; $p = 1.1 \times 10^{-6}$) from the expected proportion (Figure 5B); *cpo^{dupl/v3}* was expected to express a low-diapause phenotype of 25% or less in diapause (SI: Schmidt et al., 2008). *cpo^{del/v3}* was expected to show a high-diapause phenotype of 70% or greater in diapause, and no significant difference ($p = 0.50$) was found between observed (52.9%) and expected proportions (SI: Schmidt et al. 2008). The proportion in diapause for *cpo^{wt/v3}* and *cpo^{hypo/v3}* was measured in a different replicate (Figure 5B) – no significant difference was found between observed (100%) and expected (~90%) proportions for *cpo^{hypo/v3}* flies ($p = 0.080$), but there was a significant difference between the expected (~45%) and observed (86.5%) proportions for *cpo^{wt/v3}* flies ($p = 2.7 \times 10^{-7}$). The observed proportion in diapause changed between the two replicates for *cpo^{wt/v3}* and *cpo^{hypo/v3}* flies, but was still different between the two genotypes ($p = 0.026$). Despite inconsistencies between the observed and expected proportions in diapause of both genotype pairs, the *cpo^{dupl/v3}* and *cpo^{del/v3}* genotype pair showed an effect of diapause propensity on bacterial load that is consistent with my hypothesis, while the *cpo^{wt/v3}* and *cpo^{hypo/v3}* pair did not.

A.



B.

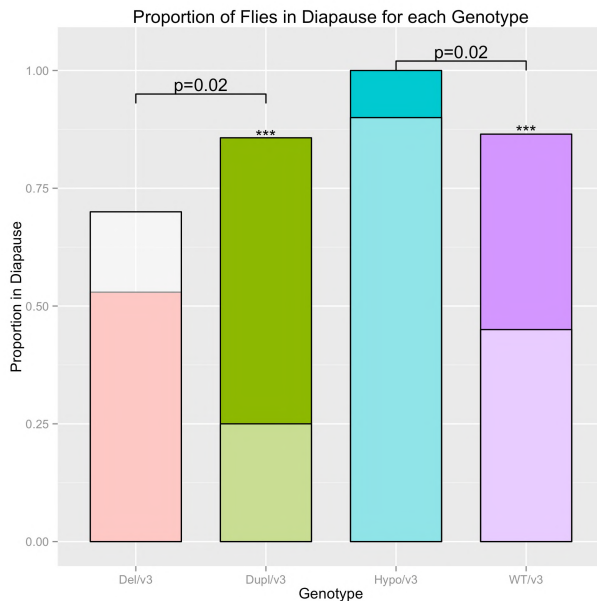


Figure 5. Infection of *cpo^{del/v3}* (Del/v3) and *cpo^{dupl/v3}* (Dupl/v3) virgin females with *P. rettgeri* and *E. faecalis* at 11°C and 10L:14D. (A) Initial time point sample size (n = 5); 96-hour time point sample size (n = 10). P-values were generated by using ANOVA on a linear model. (B) Colored bars represent the observed proportion in diapause; lighter shading/white bars represent the expected proportion in diapause (SI: Schmidt et al., 2008). *cpo^{wt/v3}* (replicate 2: n = 74), *cpo^{hypo/v3}* (replicate 2: n = 47), *cpo^{del/v3}* (n = 17), *cpo^{dupl/v3}* (n = 35). P-values were generated using a proportion test with Yates continuity correction on the observed proportions. Asterisks show a significant difference between the observed and expected proportion in diapause: * (p < 0.001)**

Effect of Chronic Infection on Entry into Diapause

Oregon-R virgin females were infected with *P. rettgeri* and *E. faecalis*, put at diapause conditions for 4 weeks, and then the diapause state was evaluated at the end of the 4 week period. 100% of flies in all the treatments (sterile needle: n = 20, CO₂: n=35, *P. rettgeri*: n=34), and *E. faecalis*: n=33) did not go into diapause.

Discussion:

I hypothesized that the diapause state would cause an increase in infection resistance in *Drosophila melanogaster*. I predicted that entry into diapause would result in an increased resistance to bacterial pathogens. The genotypes used in the infection experiments differed in their propensities to enter diapause, although these propensities were different than those due to differential expression of the *couch potato* gene that Schmidt et al. predicted (2008). This inconsistency shows that extraneous factors affecting diapause may have been introduced into this study. There was no effect of genotype for the infection carried out at 24°C and 12L:12D, which are optimal growth conditions for *D. melanogaster*. The *cpo*^{wt/v3} (wild-type) and *cpo*^{hypo/v3} (*cpo* hypomorph) genotypes did not differ in bacterial loads at diapause conditions. For the first replicate, the proportion in diapause of each genotype differed from each other and also from the expected proportions. For the second replicate, the *cpo*^{hypo/v3} genotype did not differ from the expected proportions, but the *cpo*^{wt/v3} genotype did. Even though there was a consistent difference in proportion of diapausing flies between the two genotypes, there was no difference in bacterial load observed for either replicate. Therefore, this evidence shows that entry into diapause does not result in a higher resistance.

There was a significant difference in bacterial loads for the *cpo*^{del/v3} (*cpo* deletion) and *cpo*^{dupl/v3} (*cpo* duplication) genotypes at diapausing conditions. *cpo*^{dupl/v3} showed a significantly

higher proportion of diapausing flies than $cpo^{del/v3}$, as well as a decreased bacterial load for both pathogens. Once again, the proportions of flies in diapause for the two genotypes differed significantly from expectation – $cpo^{dupl/v3}$ had a higher proportion of flies in diapause, whereas Schmidt et al. show that $cpo^{del/v3}$ has a higher proportion of flies in diapause (2008). This pair of genotypes lends support for my hypothesis that there is a connection between diapause and immunity.

The persistent inconsistencies between the observed and expected proportions of flies in diapause prevent complete confidence in the link between cpo expression, diapause, and defense. The issue likely stems from the parental lines; instead of expressing wild-type eye color, the cpo^{hypo} line expresses a brown eye color, and cpo^{v3} expresses wild-type eyes instead of scarlet. These lines all have a w⁶³²⁶ background, which has white eyes. The cpo^{hypo} and cpo^{v3} lines have a wild-type eye color marker in the P-element insertion in the cpo gene (Schmidt et al. 2008). Therefore, the eye phenotypes may be linked with cpo expression that differs from expectation, which would then affect diapause propensity. The underlying cause for the differences in the eye and diapause phenotypes is as yet undetermined. An analysis of the expression of the *couch potato* gene for the parental lines and the different hybrid genotypes would determine if any inconsistencies are due to unexpected overexpression or underexpression of the gene. As the lines are all the same background, the chance that there are some unforeseen epistatic effects is unlikely, but the inconsistent eye phenotypes suggest that problems with the genetic background could also be a possibility. To lend strong support for my hypothesis, future experiments would have to rectify this issue, so that any effects on immunity could be directly tied to differential diapause expression without the possibility of other factors (either genetic or environmental) being involved. As of now, a potential reason why the $cpo^{dupl/v3}$ and $cpo^{del/v3}$ genotype pair showed a difference in resistance and the $cpo^{wt/v3}$ and $cpo^{hypo/v3}$ pair did not could

be due to the difference in underlying genetics of the genotypes. The *cpo*^{dupl} and *cpo*^{del} lines are a *cpo* duplication/deletion pair, whereas the *cpo*^{wt} is a precise excision of the P-element in the BG line (Schmidt et al. 2008). The deletion and duplication of the entire gene could impact immune defense in a more drastic fashion than a hypomorph or the wild-type genotype. Therefore, I conclude that there does exist a positive effect of diapause on resistance, but the factors that influence whether this effect is present are so far undetermined.

Survival was assayed to see whether the genotype (and consequently, the diapause propensity) also had an effect on mortality. The mortality rate was not affected by the genotype, the pathogen, or their interaction at diapause conditions, whereas the mortality rate at 24°C and 12L:12D depended on the pathogen the flies were infected with. The overall levels of mortality were much lower at diapause conditions (Figures 2B, 4A, 4B). The bacterial loads at the second time point for the infection experiments are also lower at diapause conditions, especially for *E. faecalis*. These decreased bacterial loads contribute to the decreased mortality experienced at diapause conditions. Another possible contributing factor is that flies at diapause conditions show negligible senescence (Tatar et al. 2001). Tatar et al. showed that flies that were previously kept at 11°C for 3-week and 9-week periods had the same age-specific mortality as newly eclosed flies (2001). The diapause state essentially arrests the aging process, likely by upregulating processes that maintain the integrity of the body's tissues, of which immune defense may be one. There is no evidence to show that differential diapause propensities of *cpo*^{wt/v3} and *cpo*^{hypo/v3} affect the extent of their somatic survival – there was no significant effect of genotype on mortality post-infection, and mortality pre-infection was practically non-existent. It could be that the lack of difference in defense of these two genotypes is tied into an overall lack of difference in somatic survival, despite the difference in diapause rates. A future experiment that could elucidate how important the immune response is in preventing mortality

could look at survival post-infection of *cpo^{del/v3}* and *cpo^{dupl/v3}* genotypes, for which there is an established difference in bacterial loads. If there were a similar effect of genotype on survival and bacterial load, it would be possible to say that defense plays a role in the low mortality post-infection. If there were a genotype effect on bacterial load but not mortality, the low mortality could be attributed to an arrest in senescence as a result of other somatic processes altered during diapause, such as a decrease in metabolism and oxygen consumption (Tatar et al. 2001)

To test for whether infection affects entry into diapause, Oregon-R virgins were assayed for diapause after 4 weeks at diapause conditions with a chronic infection. None of the flies in any of the treatments went into diapause. Upon further review of the literature, it was found that there is 10 hour period post-eclosion during which the fly must be exposed to diapause conditions to induce the diapause state (Saunders et al. 1990). The Oregon-R virgins for this experiment were 5 to 9 days old and were kept at 24°C and 12L:12D from the start of eclosion, which explains why there was no expression of diapause.

Future experiments would need to look at how diapause and immunity affect insulin signaling in order to understand the molecular connection between them. My results have shown evidence that a connection exists between diapause and resistance, but more research is needed to extend the connection to the immune signaling pathways by way of a molecular mechanism. DiAngelo et al. showed that a constitutively active Toll receptor (part of the innate immune response that responds to gram-positive bacterial infection) disrupts insulin signaling at or downstream of PI3K (phosphatidylinositol 3-kinase), decreasing dAkt (protein kinase B) phosphorylation and leading to the nuclear localization of dFOXO (forkheadbox-containing protein) (2009). In *C. elegans*, insulin signaling pathway gene orthologs regulate entry into the dauer state, a state that is similar to diapause in insects. The mosquito, *Cx. pipiens*, disrupting insulin signaling stimulates fat accumulation and blocks ovarian development. A similar process

is present in *D. melanogaster*; fat accumulation is mediated by the nuclear localization of dFOXO (Hahn & Denlinger 2011). Contrary to this, DiAngelo et al. showed that disruption of insulin signaling by active Toll caused a depletion of fat reserves in the fat body. The localization of dFOXO, phosphorylation of dAkt, and triglyceride levels in the fat body need to be examined in diapausing females to determine if insulin signaling is disrupted in diapause.

These factors, as well as the level of expression of anti-microbial peptides post-infection, need to be examined in both diapausing and non-diapausing females to see what effect an active immune system has on insulin signaling and what effect the disrupted insulin signaling pathway will have on the activation of the immune system. Previous studies have shown that exposure to cold stress in *D. melanogaster* increases tolerance to fungal infection and upregulates expression of genes coding for anti-microbial peptides (Sinclair et al. 2013). Any future experiments would have to dissect whether cold stress and diapause act on the immune system through the same pathway, by different mechanisms, or whether there is cross-talk between them. The Imd immune signaling pathway is primarily responsible for defense against gram-negative bacteria; DiAngelo et al. did not show an effect of Imd activation on insulin signaling, but in my study, there was a decreased load of both gram-negative and gram-positive bacteria (DiAngelo et al. 2009). Future investigation into the connection between diapause and immunity should look at the effects of diapause and insulin signaling on both immunity pathways to find out whether these pathways have independent connections to the diapause state or if they are part of the same one. Tatar et al. showed that the length of diapause was inversely correlated with reproductive output post-diapause, with flies undergoing a 9-week diapause treatment reducing gross maternity to 28.1% of the control flies (2001). This is strong evidence that there is a trade-off between somatic maintenance and reproductive efforts. Establishing how these factors affect one another will give insight into the mechanisms behind this life history trade-off. This insight is

important because while life history trade-offs are commonly observed, little is known about the processes behind them. As the climate changes, there is potential for evolution at any of the points in this pathway; using this knowledge, we can predict how the changing climate may affect the life history and pathogen defense capabilities of the fruit fly.

In conclusion, diapause expression was correlated with higher resistance to bacterial infection. There is promising evidence that these two processes are connected by way of insulin signaling (DiAngelo et al. 2009; Hahn & Denlinger 2011). Whether that is actually the case remains to be established. Finding the connection will have implications on our understanding of life history trade-offs and the way they may be shaped by a changing climate.

Literature Cited

- DiAngelo JR, Bland ML, Bambina S, Cherry S, Birnbaum MJ. 2009. The immune response attenuates growth and nutrient storage in drosophila by reducing insulin signaling. *Proc. Natl. Acad. Sci. U. S. A.* 106:20853-20858.
- Hahn DA, Denlinger DL. 2011. Energetics of insect diapause. *Annual Review of Entomology* 56:103-121.
- R Core Team. 2013. R: A language and environment for statistical computing.
- Richard DS, Rybczynski R, Wilson TG, Wang Y, Wayne ML, Zhou Y, Partridge L, Harshman LG. 2005. Insulin signaling is necessary for vitellogenesis in drosophila melanogaster independent of the roles of juvenile hormone and ecdysteroids: Female sterility of the chico1 insulin signaling mutation is autonomous to the ovary. *J. Insect Physiol.* 51:455-464.
- Richard DS, Jones JM, Barbarito MR, Stacy Cerula, Detweiler JP, Fisher SJ, Brannigan DM, Scheswohl DM. 2001. Vitellogenesis in diapausing and mutant drosophila melanogaster: Further evidence for the relative roles of ecdysteroids and juvenile hormones. *J. Insect Physiol.* 47:905-913.
- Saunders DS, Henrich VC, Gilbert LI. 1989. Induction of diapause in drosophila melanogaster: Photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proc. Natl. Acad. Sci. U. S. A.* 86:3748-3752.

Saunders DS, Gilbert LI. 1990. Regulation of ovarian diapause in *Drosophila melanogaster* by photoperiod and moderately low temperature. *J. Insect Physiol.* 36:195-200.

Schmidt PS, Zhu C, Das J, Batavia M, Yang L, Eanes WF. 2008. An amino acid polymorphism in the couch potato gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 105:16207-16211.

Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA. 2013. Cross-tolerance and cross-talk in the cold: Relating low temperatures to desiccation and immune stress in insects. *Integr. Comp. Biol.* 53:545-556.

Tatar M, Chien SA, Priest NK. 2001. Negligible senescence during reproductive dormancy in *Drosophila melanogaster*. *The American Naturalist* 158:248-258.

Williams KD, Busto M, Suster ML, So AK-, Ben-Shahar Y, Leivers SJ, Sokolowski MB. 2006. Natural variation in *Drosophila melanogaster* diapause due to the insulin-regulated PI3-kinase. *Proc. Natl. Acad. Sci. U. S. A.* 103:15911-15915